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Evidence That Botulinum Toxin Receptors on Epithelial Cells and Neuronal Cells Are Not Identical: Implications for Development of a Non-Neurotropic Vaccine

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ABSTRACT

Botulinum toxin typically interacts with two types of cells to cause the disease botulism. The toxin initially interacts with epithelial cells in the gut or airway to undergo binding, transcytosis, and delivery to the general circulation. The toxin then interacts with peripheral cholinergic nerve endings to undergo binding, endocytosis, and delivery to the cytosol. The receptors for botulinum toxin on nerve cells have been identified, but receptors on epithelial cells remain unknown. The initial toxin binding site on nerve cells is a polysialoganglioside, so experiments were performed to determine whether polysialogangliosides are also receptors on epithelial cells. A series of single mutant and dimutant forms of the botulinum toxin type A binding domain (HC_{50}) were cloned and expressed. One of these (dimutant HC_{50} $A_{W1266L,Y1267S}$) was shown to have lost its ability to bind nerve cells (phrenic nerve-hemidiaphragm preparation), yet it retained its ability to bind and cross human epithelial monolayers (T-84 cells). In addition, the wild-type HC_{50} and the dimutant HC_{50} displayed the same ability to undergo binding and transcytosis (absorption) in a mouse model. The fact that the dimutant retained the ability to cross epithelial barriers but did not possess the ability to bind to nerve cells was exploited to create a mucosal vaccine that was non-neurotropic. The wild-type HC_{50} and non-neurotropic HC_{50} proved to be comparable in their abilities to: 1) evoke a circulating IgA and IgG response and 2) evoke protection against a substantial challenge dose of botulinum toxin.

Introduction

Botulinum toxin (BoNT) is a microbial protein that causes a potentially fatal neuroparalytic disease called botulism (Schiavo et al., 2000). The disease can occur in several different variants, but the most common is oral poisoning. Patients can ingest food contaminated with preformed toxin (primary intoxication), or they can ingest food contaminated with organisms that manufacture toxin in situ (primary infection with secondary intoxication). Although less common, botulism can also occur as a form of inhalation poisoning (Holzer, 1962). In this case, it is only primary intoxication that is known to exist as a natural disease.

Oral poisoning and inhalation poisoning have in common that there are two sequences of events that lead to an adverse outcome. During the first sequence of events, BoNT is absorbed into the body (Simpson, 2004). More precisely, the toxin binds to the apical surface of epithelial cells in the gut or airway (namely, transport cells) (Ahsan et al., 2005). This is followed by receptor-mediated endocytosis, transcytosis, and eventual release of unmodified toxin into the general circulation (Maksymowych and Simpson, 1998; Maksymowych et al., 1999). The toxin is distributed throughout the periphery, where it binds with high affinity to the junctional region of cholinergic nerve endings (namely, target cells). This initiates the second sequence of events, which includes receptor-mediated endocytosis, pH-induced translocation to the cytosol, and enzymatic cleavage of polypeptides that govern transmitter release (Schiavo et al., 2000). Cleavage of these substrates, with the resulting blockade in exocytosis, produces the neuroparalytic outcome that is characteristic of the disease botulism.

The fact that BoNT must bind to both epithelial cells and neuronal cells raises the possibility that receptors on the two

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ABBREVIATIONS: BoNT , botulinum toxin; TPGS, D-α-tocopheryl polyethylene glycol succinate; PBST, phosphate-buffered saline with 0.05% Tween 20; NFDM, nonfat dry milk.

cell types could be similar or even identical (Couesnon et al., 2009). In the case of nerve cells, there has been significant progress in terms of identifying binding sites. Cholinergic nerve endings are thought to have two fundamentally different receptors (Montecucco, 1986). The first, which is a nonprotein receptor, brings the toxin into the plane of the membrane. The second, which is a protein receptor, is linked to subsequent events in neuroparalysis, including the phenomenon of receptor-mediated endocytosis.

The putative identity of the nonprotein binding site was first proposed many years ago (Simpson, 1981). A series of in vitro and in vivo studies suggested that polysialogangliosides were involved in the binding of several toxin serotypes. More recent work involving inhibitors of complex ganglioside synthesis (Yowler et al., 2002) and genetic engineering to eliminate complex gangliosides (Bullens et al., 2002) has confirmed the role of these lipids.

In a related line of research, investigators have determined the three-dimensional structures of three toxin serotypes [A (Lacy and Stevens, 1998), B (Swaminathan and Eswaramoorthy, 2000), and E (Kumaran et al., 2009)]. In each case the toxin is composed of three somewhat independent lobes that represent a light chain (approximately 50,000 Da), the amino-terminal portion of the heavy chain (approximately 50,000 Da), and the carboxyl-terminal portion of the heavy chain (approximately 50,000 Da). It is the latter that plays a key role in binding to nerve terminals, and it is this portion of the molecule that displays affinity for gangliosides. Thus, Rummel et al. (2004) have demonstrated that point mutations in the carboxyl-terminal portion of the toxin molecule significantly diminish binding to nonprotein receptors. Unfortunately, the amino acids that govern toxin binding to protein receptors have not yet been identified.

In the recent past, a series of studies have been performed in an effort to better characterize the phenomenon of toxin binding and penetration of gut and airway epithelial cells. This work has demonstrated that the entire light chain as well as the amino-terminal portion of the heavy chain can be removed from the holotoxin, and the residual carboxyl-terminal half of the heavy chain (HC_{50}) retains the ability to cross epithelial barriers (Maksymowych and Simpson, 2004). In the report that follows the HC_{50} domain has been used to compare the binding properties of receptors in epithelial cells with those in neuronal cells. Point mutations were introduced into the molecule at sites that were previously shown to diminish ganglioside binding. However, unlike the previous work, the present study used multiple mutations to essentially abolish neuronal binding. This approach has resulted in two noteworthy findings. First, HC_{50} domains that have been engineered to lose their affinity for polysialogangliosides and nerve endings retain a substantial portion of their affinity for epithelial cells. Second, the altered HC_{50} domains can be exploited to create a novel class of mucosal botulinum vaccines that are non-neurotropic (Simpson, 2009).

Materials and Methods

Materials. All restriction enzymes, T_4 DNA ligase, and Taq DNA polymerase were purchased from Promega (Madison, WI). Oligonucleotide primers for site-specific mutagenesis were purchased from Stratagene (La Jolla, CA). BoNT type A (BoNT/A) was purchased from Metabiologics (Madison, WI). All chemicals were of analytical grade and obtained from commercial sources.

Plasmid Construction and Bacterial Expression of HC_{50} Domains. The gene portion encoding the HC_{50} fragment of BoNT/A (strain 62A, amino acids 861-1296) was cloned into 6×His vector pQE30 (QIAGEN, Valencia, CA), yielding the expression plasmid pQEHC_{50}A.

Escherichia coli [BL21-codon plus(DE3)-RIL (Stratagene)] was used as the host strain for the expression of HC_{50} domains. Cells were grown in Terrific broth (1.2% peptone, 2.4% yeast extract, 0.94% K_2HPO_4, and 0.22% KH_2PO_4) (Difco, Detroit, MI) at 37°C, with shaking to an A_{600} of 0.6 to 0.8. Isopropyl-D-thiogalacto-pyranoside at a final concentration of 0.5 mM was added, and incubation was continued for approximately 12 h at 25°C. Bacteria from 1 liver of induced culture was harvested by centrifugation (6,000*g*, 15 min) at 4°C.

Gene Modification by Site-Directed Mutagenesis. Three single mutants and one dimutant of BoNT/A HC_{50} were generated. Point mutations were introduced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The mutagenic primers used were: forward 5′-tttataggattt***tgg***cagtttaataat-3′ and reverse 5′-attattaaactg***cca***aaatcctataaa-3′ for H1253W; forward 5′-gtagcaagtaat***ttg***tataatagacaa-3′ and reverse, 5′-ttgtctattata***caa***attacttgctac-3′ for W1266L; forward, 5′-gcaagtaattgg***tct***aatagacaaata-3′ and reverse, 5′-tatttgtctatt***aga***ccaattacttgc-3′ for Y1267S; forward, 5′-gcaagtaat***ttgtct***aatagacaaata-3′and reverse, 5′-tatttgtctatt***agacaa***attacttgc-3′ for double mutant (W1266L and Y1267S). Bold italic type indicates the locations of the point mutations.

Purification of Protein. Bacterial cells were suspended in 200 ml of the bacterial protein extract reagent B-PER (Pierce Chemical, Rockford, IL) at 4°C. Lysozyme (Sigma-Aldrich, St. Louis, MO) at a final concentration of 0.1 mg/ml, DNase (Sigma-Aldrich) at a final concentration of 0.01 mg/ml, and protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany) were added to the cell suspension and incubated on a rotating shaker for 2 h. Four hundred milliliters of 50 mM sodium phosphate containing 300 mM NaCl, pH 8.0, was added to the lysed cell suspension and allowed to stand for 30 min. The suspension was centrifuged at 27,000*g* for 40 min to remove precipitate.

The clear supernatant was loaded onto a 5-ml column of Ni-NTA superflow (QIAGEN), which was equilibrated with 50 mM sodium phosphate containing 300 mM NaCl, pH 8.0. The column was washed with 50 volumes of washing buffer (50 mM sodium phosphate containing 300 mM NaCl, and 20 mM imidazole, pH 8.0). Bound protein was eluted from the column with a gradient of increasing imidazole (100 ml of 50 mM sodium phosphate containing 300 mM NaCl and 20 mM imidazole, and 100 ml of 50 mM sodium phosphate containing 300 mM NaCl and 250 mM imidazole, pH 8.0). The active fractions (at ~100 mM imidazole) were pooled and dialyzed against 50 mM sodium phosphate, pH 6.8. The dialysate was centrifuged at 27,000*g* for 30 min to remove precipitate.

The clear supernatant was loaded onto a 4-ml cation exchange column of CM Sepharose Fast Flow (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) equilibrated with 50 mM sodium phosphate, pH 6.8. The column was washed with 50 volumes of 50 mM sodium phosphate, pH 6.8. Bound protein was eluted from the column with a gradient of increasing NaCl (50 ml of 50 mM sodium phosphate and 50 ml of 50 mM sodium phosphate with 500 mM NaCl, pH 6.8). The active fractions (at ~200 mM NaCl) were pooled and dialyzed against 50 mM sodium phosphate, pH 7.4. The purity of HC_{50}A protein was confirmed on 10% SDS-polyacrylamide gel electrophoresis and found to be more than 98% homogeneous. Approximately 15 to 20 mg of pure HC_{50}A was obtained from 1 liter of bacterial culture. Proteins were further confirmed by Western blot analysis using rabbit polyclonal antibodies raised against the heavy chain component of BoNT/A.

Cell Culture. T-84 human epithelial cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (1 g/l D-glucose) and

Ham's F-12 nutrient medium supplemented with 5% newborn calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 8 μg/ml ampicillin, and 15 mM HEPES. Cultures were maintained at 37°C in 6% CO_2. T-84 cells were fed every other day and passaged (1:2) when 95% confluent, approximately every 4 to 5 days. Passages 75 through 85 were used for experiments described in this article.

Assay for Transcytosis. The assay for BoNT and HC_{50} transport across epithelial cells was carried out essentially as described previously (Maksymowych and Simpson, 2004). In brief, T-84 cells were plated at confluent density (approximately 1.5×10^5 cells) into Transwells with 0.5 ml of medium in the upper chamber and 1.0 ml in the lower chamber. Membrane integrity and potency of tight junctions were confirmed by measuring transepithelial electrical resistance using an epithelial volt-ohmmeter (WPI, Sarasota, FL).

The assay was started by adding 0.5 ml of medium with 0.2% serum in the upper chamber and phosphate-buffered saline in the lower chamber. Transcytosis was initiated by adding $HC_{50}A$ (1×10^{-8} M) to the upper chamber and incubating for 18 h at 37°C. The entire contents of three wells from the lower chamber were collected and concentrated in Centricon 10s microconcentrators to a final volume of 50 μl, of which 15 μl/lane was loaded onto 10% SDS polyacrylamide gels.

Samples for Western blot analysis were separated according to Laemmli (1970). Proteins were transferred to nitrocellulose membranes and detected with antibodies against $HC_{50}A$ that were generated in our laboratory.

Neuromuscular Bioassay. Murine phrenic-nerve hemidiaphram preparations were excised and placed in tissue baths to bioassay the toxicity of botulinum toxin in biological specimens (Kiyatkin et al., 1997). Tissues were suspended in physiological buffer that was aerated with 95% O_2 and 5% CO_2. Phrenic nerves were stimulated continuously (0.2 Hz; 0.1- to 0.3-ms duration), and muscle twitch was recorded. Toxin-induced paralysis was measured as a 90% reduction in muscle twitch response to neurogenic stimulation.

The ability of native and mutant forms of the HC_{50} domains to compete for toxin binding sites was bioassayed with phrenic nerve-hemidiaphragm preparations. Tissues were suspended in physiological medium at 37°C, and neurogenic responses were monitored for approximately 60 min (i.e., baseline). Temperature was then lowered to 10°C to substantially abolish the active process of receptor-mediated endocytosis, and nerve stimulation was halted. Various concentrations of the HC_{50} polypeptides were added (see *Results*), and incubation was continued for 60 min. BoNT was then added, and incubation was continued for an additional 45 min. The baths were washed three times to remove all unbound HC_{50} and BoNT, temperature was raised to 37°C, and nerve stimulation was reinitiated. The paralysis times for control tissues (toxin alone) and experimental tissues (HC_{50} polypeptide plus toxin) were monitored. The goal of the assays was to identify a concentration of HC_{50} that would double the amount of time necessary for homologous holotoxin to paralyze transmission.

Animals. BALB/c mice (female, 18–20 g) were purchased from Charles River Laboratories Inc. (Wilmington, MA). All animals were housed in the animal care facility at Thomas Jefferson University and allowed unrestricted access to food and water. All procedures involving animals were reviewed and approved by the Thomas Jefferson University Institutional Animal Care and Use Committee.

Pharmacokinetics. The $HC_{50}A$ polypeptide and the dimutant polypeptide ($HC_{50}A_{W1266L,Y1267S}$) were administered via the inhalation route. This route of exposure was selected over the oral route to obviate the problems associated with digestion of antigens in the gut. The HC_{50} domains were administered as a single dose (20 μg in 20 μl of PBS) to mice that were lightly anesthetized with isoflurane (Isothesia; Abbott Laboratories, Abbott Park, IL). The bodies of animals were maintained in a semihorizontal position to minimize drainage into the posterior pharynx. Mice were sacrificed at different time points (six or more mice per time point), and the total amount of antigen in serum was measured.

Immunoassay for Polypeptides. The HC_{50} domain was quantified with a luminescent sandwich immunoassay by using a human monoclonal antibody as a capture device (mAb 6A; Adekar et al., 2008) and rabbit polyclonal antibodies as part of a reporter device. Monoclonal antibodies were diluted in phosphate-buffered saline to a concentration of 3 μg/ml and coated on black Nunc Maxisorp plates (Nalge Nunc International, Rochester, NY). Plates were covered and stored overnight at 4°C, then antigen solution was aspirated and discarded. Plates were blocked with 300 μl per well of 2% nonfat dry milk (NFDM) in phosphate-buffered saline with 0.05% Tween 20 (PBST) for 1 h at 37°C. Blocking solution was aspirated and plates were washed three times with PBST.

Standards and experimental plasma samples were diluted 1:1 in phosphate-buffered saline, then 50 μl per well of diluted samples was added in triplicate to plates. Plates were covered and shaken slowly at room temperature for 1 h, then washed three times with PBST. Affinity-purified, biotinylated polyclonal anti-heavy chain antibodies were diluted in NFDM/PBST to a concentration of 3 μg/ml and added to plates (50 μl per well). Plates were incubated for 1 h at 37°C, then washed three times with PBST. A streptavidin poly-horseradish peroxidase conjugate was diluted to a concentration of 300 ng/ml in NFDM/PBST and added to plates (50 μl per well). Plates were incubated for 30 min at 37°C, followed by nine washes with PBST. A luminol substrate (Thermosci SuperSignal ELISA Femto Substrate; Thermo Fisher Scientific, Waltham, MA) was added to plates, and relative luminescence values were measured with Biotek Synergy 2 Luminometer (BioTek Instruments, Winooski, VT).

Vaccination Protocol. Antigen was administered by the intranasal route. Each protein (20 μg) was administered by a single application of a 20-μl phosphate-buffered saline solution (pH 7.0) to the nares. In certain experiments the animals received antigen in the presence of an adjuvant (vitamin E TPGS; 1.0%, w/v). During a standard protocol, mice were vaccinated at times 0, 2, and 4 weeks. At 6 weeks, aliquots of blood were obtained for quantitation of IgA and IgG, and animals were challenged (see below).

Enzyme-Linked Immunosorbent Assay. Antibody titers in mouse serum were determined by standard procedures. In brief, flat-bottom, 96-well Corning plates (Corning Glassworks, Corning, NY) were coated with the HC_{50} domains of botulinum toxin (300 ng/well) and incubated at 4°C overnight, followed by washing with phosphate-buffered saline plus Tween (0.1%) pH 7.4. The plates were blocked with 1% bovine serum albumin. Two-fold serial dilutions of serum samples were added to the plates and incubated at 37°C for 60 min. IgG titers were determined using peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich), and IgA titers were determined using peroxidase-conjugated goat anti-mouse IgA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Secondary antibodies were diluted 1:1000 in phosphate-buffered saline. The primary and secondary antibodies were incubated for 30 min at 37°C, after which 2, 2′-azino-bis(3-ethylbenthiazoline-6-sulfonic acid) was added as a substrate, and the plates were incubated for an additional 30 min at 37°C. Endpoint titers were determined as the reciprocal of the last dilution yielding an absorbance at 405 nm that was above the control value (preimmune serum).

Challenge with Botulinum Toxin. The most characteristic outcome of toxin action is muscle weakness and paralysis. This outcome is easily discernible as decreased locomotion and ultimately as a failure of respiration. During challenge experiments, animals received large doses of toxin that typically produce respiratory failure and death within 1 to 2 h. To minimize pain and suffering, animals were not allowed to die from paralysis of respiration. Instead, animals were observed throughout the protocols. When signs of neuromuscular weakness became obvious (i.e., absence of locomotor activity) animals were sacrificed in accordance with guidelines from the Association for Assessment and Accreditation of Laboratory Animal Care (e.g., CO_2).

Results

Cloning, Expression, and Purification of Wild-Type and Mutant HC_{50} Domains. The three-dimensional structures of several botulinum toxins have been determined (Lacy and Stevens, 1998; Swaminathan and Eswaramoorthy, 2000; Kumaran et al., 2009), and the presumed ganglioside binding pockets have been identified. This in turn has led to the identification of specific amino acids in the carboxyl terminus of the toxin molecule that participate in neuronal ganglioside binding. Site-directed mutagenesis of these residues leads to variable reductions in binding to a prototype ganglioside (GT1b) and a prototype neuronal preparation (phrenic nerve-hemidiaphragm; Rummel et al., 2004).

In the present study, the wild-type HC_{50} domain, as well as a series of single mutant and double mutant variations of the BoNT/A HC_{50} domain, were generated (Fig. 1). For the wild-type domain, a typical yield was approximately 15 mg/liter. The final product was essentially homogeneous (Fig. 2).

Site-directed mutagenesis was performed as described under *Materials and Methods*, and the location and nature of the intended mutations was confirmed by DNA sequencing. All of the single mutants and dimutants were expressed and purified by the same techniques used for wild-type polypeptides. The yields were closely similar to those obtained with the wild-type HC_{50} domains (Fig. 2).

Bioassay of Wild-Type HC_{50} Activity Using the Phrenic Nerve-Hemidiaphragm Preparation. The most sensitive site for botulinum toxin activity in the periphery is the cholinergic neuromuscular junction. The isolated HC_{50} domain retains affinity for neuromuscular junctions, and this affinity is typically quantified by measuring the ability of the polypeptide to antagonize the actions of the parent toxin. This functional assay ensures that the HC_{50} activity being quantified is relevant to the neuroparalytic actions of the parent toxin (Lalli et al., 1999; Yang et al., 2004).

A series of dose-response experiments were done to determine an appropriate test concentration of wild-type BoNT/A HC_{50} that could serve as a comparator for subsequent work. In all cases, the test concentration of the parent toxin was

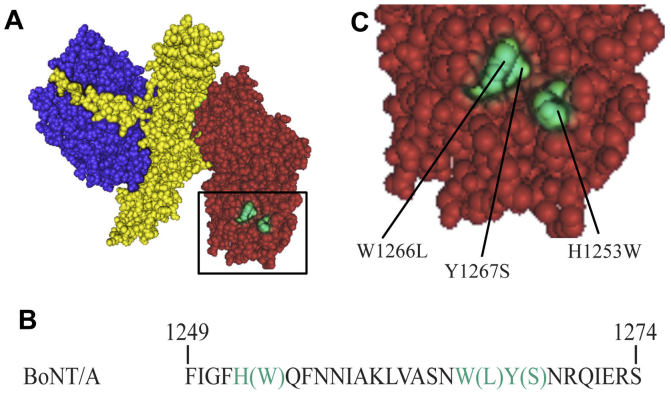

Fig. 1. BoNT/A is composed of three globular domains (A): the light chain (blue), the amino-terminal half of the heavy chain (yellow), and the carboxyl-terminal half of the heavy chain (red). Three amino acids in the carboxyl-terminal half of the heavy chain (HC_{50}) have been implicated in toxin binding to gangliosides: His1253, Trp1266, and Tyr1267 (green). The general location of the mutations in the carboxyl-terminal half of the heavy chain is shown in A. The specific location of these mutations in the linear sequence of the toxin is shown in B. The specific location of the mutations in the conformational structure of the heavy chain is shown in C (which represents the boxed area in A).

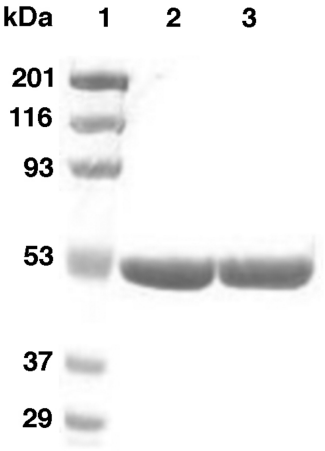

Fig. 2. The wild-type and mutant forms of BoNT/A HC_{50} were expressed and isolated as described under *Materials and Methods*. When examined by SDS-polyacrylamide gel electrophoresis, the various preparations were essentially homogeneous and comparable in apparent molecular mass. The results shown were obtained with wild-type BoNT/A HC_{50} and the dimutant ($HC_{50}/A_{W1266L,\ Y1267S}$). Lane 1, molecular mass standards. Lane 2, wild-type HC_{50} (3 μg). Lane 3, dimutant HC_{50} (3 μg).

10^{-12} M. In the absence of HC_{50}, the average time for BoNT/A to paralyze transmission was 146 ± 13 min.

Various concentrations of BoNT/A HC_{50} were tested in an attempt to find one that would increase paralysis times caused by toxin by at least 2-fold. A doubling of paralysis times is approximately equivalent to a reduction in apparent toxicity of one order of magnitude. The results, which are illustrated in Fig. 3, indicated that an HC_{50} concentration of 5×10^{-8} M produced the desired outcome.

Bioassay for Mutant HC_{50} Activity at the Neuromuscular Junction. Point mutations were introduced into the

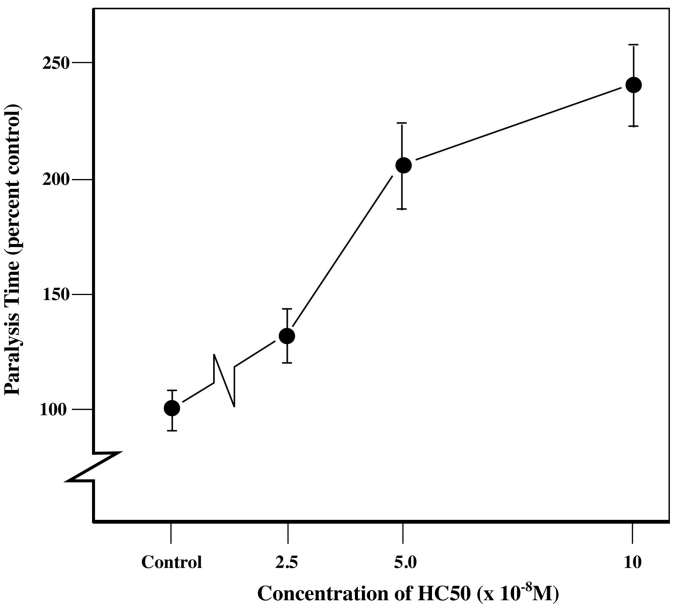

Fig. 3. Murine phrenic nerve-hemidiaphragm preparations were incubated with BoNT/A (10^{-12} M) and various concentrations of the HC_{50} domain from this toxin (n = 3 or more per data point). The average paralysis time of tissues treated with toxin alone was 146 ± 13 min. In the presence of 5×10^{-8} M HC_{50} polypeptide, the paralysis times of tissues increased approximately 2-fold. This concentration was selected for all subsequent experiments with single mutant and dimutant forms of the polypeptide.

ganglioside binding regions of the BoNT/A HC_{50} domain, and the ability of these mutants to antagonize the actions of the parent toxin was determined. The procedures were identical to those used with the wild-type polypeptide. The three BoNT/A single mutants (H1253W, W1266L, and Y1267S) all had substantially diminished ability to antagonize the parent toxin (Table 1). Thus, the percentage increases in toxin-induced paralysis times with the mutants were less than that seen with the wild-type polypeptide (10–42% for single mutants versus 105% for wild type).

TABLE 1
Paralysis times for phrenic nerve-hemidiaghragm preparations

Recombinant Polypeptides[a]	Paralysis Time[b]	Increase in Paralysis Time
	min ± S.E.M.	%
BoNT/A only	146 ± 13	0
BoNT/A + $HC_{50}A$	300 ± 21	105
BoNT/A + $HC_{50}A_{H1253W}$	207 ± 17	42
BoNT/A + $HC_{50}A_{W1266L}$	161 ± 17	10
BoNT/A + $HC_{50}A_{Y1267S}$	171 ± 14	17
BoNT/A + $HC_{50}A_{W1266L, Y1267S}$	139 ± 12	0

[a] The BoNT/A concentration was 1×10^{-12} M ($n = 3$ or more per group). The wild-type and single mutants of BoNT/A HC_{50} were tested at a concentration of 5×10^{-8} M. The dimutant HC_{50} was tested at a concentration of 5×10^{-7} M.
[b] Amount of time required for toxin to cause 90% reduction in twitch amplitude.

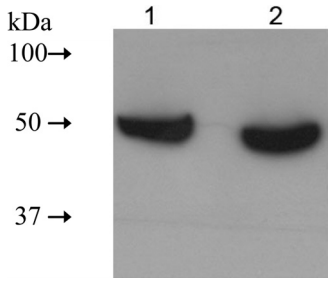

Fig. 4. The wild-type (lane 1) and dimutant (lane 2) forms of BoNT/A HC_{50} were added to the apical surface of epithelial monolayers at a concentration of 10^{-8} M, and 18 h later the contents of the basal chamber were assayed by Western blot analysis of polyacrylamide gels. The results indicated that the two polypeptides possessed comparable abilities to bind and penetrate epithelial barriers.

To ensure that binding to neuromuscular junctions was essentially abolished a dimutant of the HC_{50} domain was generated by combining W1266L and Y1267S. When tested at the same concentration as that used with wild-type and single mutants, the dimutant provided no detectable protection against the parent toxin. When the dimutant was tested at a concentration that was 10-fold higher, there was still no detectable protection against the holotoxin (Table 1).

Transcytosis of Wild-Type and Dimutant Forms of BoNT/A HC_{50} Domains. The minimum essential domain for BoNT binding to epithelial cells is located within the HC_{50} domain (Maksymowych and Simpson, 2004). Therefore, the respective abilities of wild-type and dimutant HC_{50} domains to penetrate human epithelial monolayers were examined. Both polypeptides were added to the apical surfaces of epithelial cells at a concentration of 10^{-8} M. Aliquots were subsequently collected from the basal chamber of the Transwell apparatus, and these aliquots were examined by Western blot analysis. As shown in Fig. 4, the wild-type and dimutant forms of BoNT/A HC_{50} crossed epithelial barriers, and the extent of transcytosis for the two was comparable.

Evidence for In Vivo Binding and Transcytosis. The wild-type and dimutant forms of $HC_{50}A$ were administered by the intranasal route to mice (20 μg per mouse), and at various times thereafter blood was collected and plasma samples were assayed. As shown in Fig. 5, both polypeptides underwent binding and transcytosis across airway epithelial barriers to reach the general circulation. Furthermore, the rate and extent of absorption of the wild-type and dimutant HC_{50} domains were closely similar. There was no evidence that loss of ganglioside binding by the dimutant produced loss of binding and transcytosis in vivo.

Vaccination with Dimutant Forms of HC_{50}. The HC_{50} domains of several serotypes of BoNT have been shown to be efficacious vaccines against their respective parent toxins (Simpson, 2009). Initially, these polypeptides were shown to be immunogens by the injection route, but, more recently, they have been shown to be active by the mucosal (inhalation) route (Ravichandran et al., 2007).

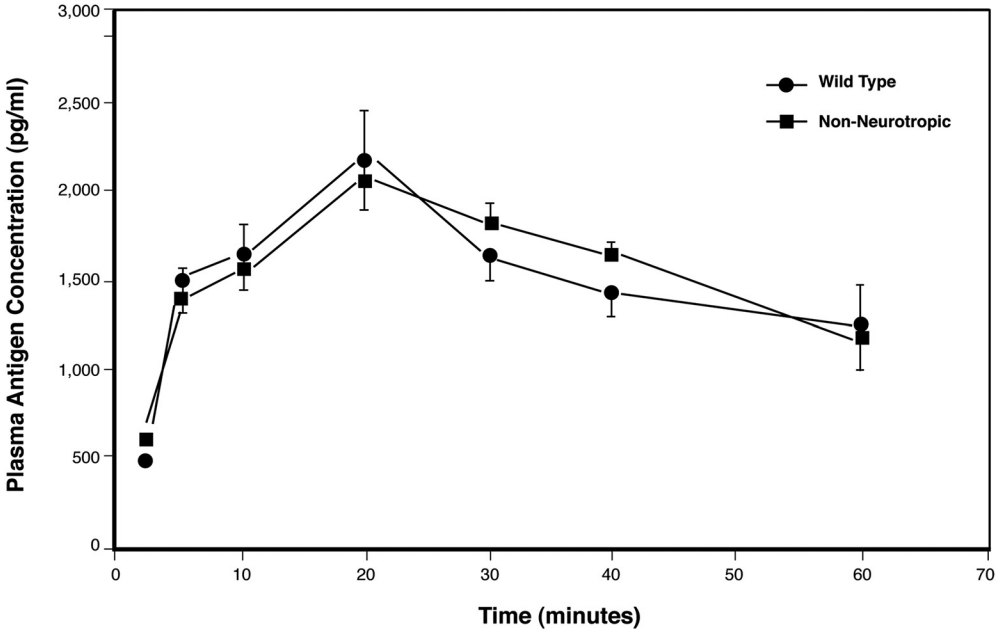

Fig. 5. Wild-type BoNT/A HC_{50} or the dimutant variant were administered as a single dose (20 μg in 20 μl of phosphate-buffered saline) by the intranasal route. Mice were bled retro-orbitaly (350 μl) at different time points (six or more mice at each time point), and the total amount of polypeptide in plasma samples was measured by luminescent immunoassay. The two polypeptides displayed almost identical rates and amounts of absorption.

In the experiments described above, the dimutant form of BoNT/A was found to retain its in vitro and in vivo ability to bind and penetrate epithelial barriers, whereas this same molecule had lost its ability to bind to cholinergic nerve endings. This raises the possibility that the dimutant form of HC_{50} could be a mucosal vaccine that is non-neurotropic. To test this possibility, animals were vaccinated with the dimutant polypeptide, then challenged with toxin.

Mice ($n = 10$) were given the dimutant polypeptide by the inhalation route (20 μg), as described under *Materials and Methods*. Two weeks after the final booster, aliquots of blood were obtained, and enzyme-linked immunosorbent assay titers for circulating IgA and IgG were determined. As shown in Fig. 6A, the dimutant form of BoNT/A HC_{50} evoked circulating immunoglobulin responses. The magnitudes of these responses were similar to those previously reported for BoNTA wild-type HC_{50} (Ravichandran et al., 2007).

The magnitudes of the immunoglobulin responses are well within the range that is associated with resistance to poisoning. This premise was confirmed by challenging control animals and vaccinated animals with 10^3 mouse LD_{50} of the parent toxin (Table 2). As expected, all control animals became visibly ill within approximately 100 min. All vaccinated animals survived for more than 4 days, and there were no signs of illness in any of these animals.

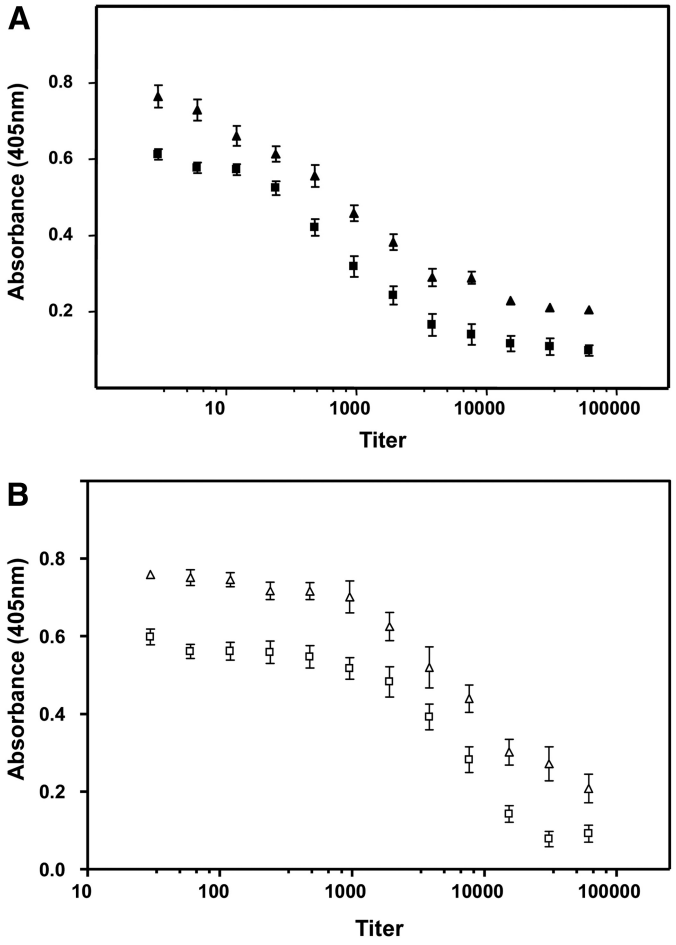

Fig. 6. A, mice ($n = 10$) were vaccinated with the dimutant from BoNT/A HC_{50}. At the end of the protocol (day 42), serum samples were obtained and assayed for circulating IgA (■) and IgG (▲). The results demonstrated that the dimutant polypeptide evoked a significant response with both immunoglobulins. B, mice ($n = 10$) were vaccinated with the dimutant from BoNT/A HC_{50} domain plus adjuvant (vitamin E-TPGS; 1%, w/v). On day 42, serum samples were obtained and assayed for immunoglobulin responses. The data show that the adjuvant helped to evoke IgA (□) and IgG (△) responses that were greater than those in the absence of adjuvant (i.e., A).

TABLE 2
Resistance to challenge with botulinum toxin type A

Vaccination Paradigm[a]	Challenge Dose	Survival
	LD_{50}	
Control	10^3	0/10
$HC_{50}A$	10^3	10/10[b]
$HC_{50}A_{W1266L, Y1267S}$	10^3	10/10[b]
Control	10^4	0/10
$HC_{50}A$[c]	10^4	10/10[b]
$HC_{50}A_{W1266L, Y1267S}$[c]	10^4	10/10[b]

[a] BoNT/A was administered by the intraperitoneal route ($n = 10$ per group).
[b] Animals that survived displayed none of the signs of botulism.
[c] Antigen plus adjuvant (vitamin E-TPGS).

Vaccination with Dimutant Forms of HC_{50} Plus an Adjuvant (Vitamin E). Two neutraceuticals (vitamin E-TPGS and chitosan) have previously been shown to act as adjuvants to boost the magnitude of responses to wild-type HC_{50} (Ravichandran et al., 2007). Therefore, an effort was made to determine whether the responses to dimutant forms of HC_{50} could also be enhanced.

Mice ($n = 10$) were vaccinated with BoNT/A dimutant HC_{50} as described above, except that the antigen was administered with vitamin E-TPGS. As shown in Fig. 6B, the circulating IgA response in the presence of adjuvant was considerably greater than that in the absence of adjuvant (compare with Fig. 6A). The IgG response, whether in the absence or presence of adjuvant, was substantial, but it was elevated still further by adjuvant.

Control animals and vaccinated animals were challenged with 10^4 LD_{50} of BoNT/A (Table 2). As expected, all control animals became visibly ill within less then 100 min. All vaccinated animals survived, and there were no signs of illness in any of those animals.

Neutralizing Antibodies and Neuromuscular Blockade. When delivered by the mucosal route, the wild-type HC_{50} domain evokes three levels of antibody-mediated protection: 1) blockade of toxin absorption by epithelial cells, 2) enhanced clearance of toxin from the general circulation, and 3) blockade of toxin binding to neuronal cells (Ravichandran et al., 2007). The fact that the dimutant HC_{50} domain has lost affinity for cholinergic nerve endings raises the question of whether anti-mutant HC_{50} antibody would still possess the third mechanism for neutralization, i.e., the ability to block toxin binding to neuronal cells.

Antiserum (0.1 ml) from mice that had been vaccinated with wild-type HC_{50} or dimutant HC_{50} was incubated with toxin (3×10^{-12} M) for 60 min at room temperature, after which the mixtures were added to phrenic nerve-hemidiaphragm preparations. The results, which are illustrated in Fig. 7, show that control tissues (toxin alone) were paralyzed within approximately 120 min. However, experimental tissues (toxin previously incubated with antiserum) did not paralyze. Both wild-type HC_{50} and dimutant HC_{50} evoked antibodies that protected nerve endings from the effects of toxin. Thus, the introduction of mutations into the HC_{50} domain can: 1) cause the polypeptide to lose its ability to bind to the neuromuscular junction, but 2) not cause the polypeptide to lose its ability to evoke antibodies that block toxin action at the neuromuscular junction.

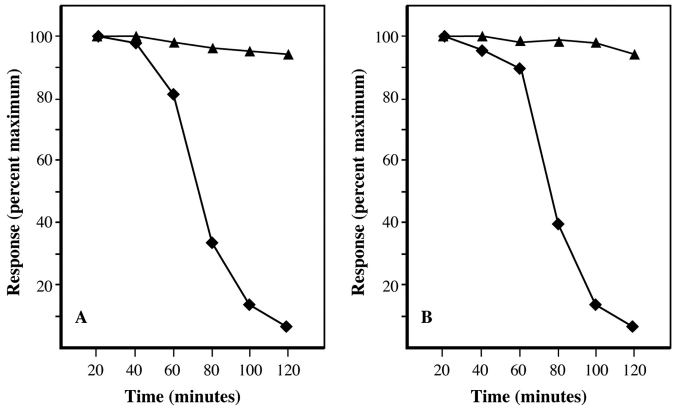

Fig. 7. BoNT/A (3×10^{-12} M) was incubated alone (♦) or in the presence of antiserum (0.1 ml) obtained from mice vaccinated with wild-type HC_{50} (▲; A) or the dimutant HC_{50} (▲; B). Incubation was for 60 min at room temperature, after which mixtures were added to phrenic nerve-hemidiaphragm preparations. Control tissues (toxin alone) were paralyzed within 120 min, whereas experimental tissues (toxin plus antiserum) were not paralyzed, even when monitored for 240 min.

Discussion

There has been considerable progress in the effort to identify neuronal receptors for botulinum toxin, including serotype A. This progress has been made in the context of a widely held belief that each serotype actually has two classes of receptors (Montecucco, 1986). For most serotypes, including serotype A, the initial nonprotein binding is likely to be mediated by a polysialoganglioside (see *Introduction*). This may explain why lectins that have affinity for sialic acid residues can block the neuroparalytic actions of most toxin serotypes (Bakry et al., 1991). The situation seems to be more complex for the subsequent protein binding, because these receptors may be somewhat serotype-specific. For example, the neuronal protein receptor for BoNT/A is reported to be synaptic vesicle protein 2, whereas this is not the receptor for serotype B (Dong et al., 2006).

In contrast to the work on neuronal receptors, little is known about epithelial receptors (Maksymowych and Simpson, 1998). Nevertheless, one might reasonably wonder whether neuronal receptors and epithelial receptors could be similar or even identical. In an effort to address this possibility, this article has focused on the initial nonprotein binding. This focus was selected because: 1) several serotypes, including A, share an affinity for gangliosides, and 2) specific regions and even specific amino acids that govern ganglioside binding have been identified.

The general strategy that has been used is introduction of mutations into the HC_{50} domain of BoNT/A, with the intent of eliminating neuronal binding. These mutants were then examined to determine whether they retained any measurable ability to bind and penetrate epithelial barriers. If binding and transcytosis in epithelial cells were to be lost, this could mean that neuronal and epithelial receptors are similar or identical. Alternatively, if binding and transcytosis were retained, this would suggest that the two classes of receptors are not identical.

Aside from revealing an important characteristic of botulinum toxin receptors, a discovery of nonidentity would carry important clinical implications. Most work aimed at developing a vaccine against botulinum toxin has used the HC_{50} domain as an antigen (Simpson, 2009). This polypeptide does evoke an immune response, but it also carries a potential risk. The HC_{50} domain can bind and enter nerve endings (Lalli et al., 1999), and this is certainly not a desirable characteristic of a vaccine candidate. Therefore, alterations in the HC_{50} domain that abolish neuronal binding but that do not abolish epithelial transport or immunogenicity would represent a substantial advance in vaccine development.

Assessment of Binding Activity. The wild-type BoNT/A HC_{50} domain, as well as a group of single mutant and dimutant derivatives of this domain, were cloned, expressed, and purified. The levels of expression and efficiencies of purification for the mutants were comparable with those of the native protein. In addition, gel analyses (Western) indicated that the molecular weights and the immunoreactivities of the mutants were indistinguishable from that of the native protein.

The phrenic nerve bioassay was used to gauge the residual affinity of mutants for cholinergic nerve endings, which are the target cells for botulinum toxin action. Single mutants displayed variable losses in affinity, but the dimutant seemed to have lost any meaningful ability to bind to nerve endings. Thus, a dimutant of BoNT/A HC_{50} (W1266L and Y1267S) had no measurable ability to occlude receptors and protect neuromuscular junctions against the actions of native toxin. This result was obtained even when using a large molar excess of the dimutant.

The dimutant was also evaluated for its ability to bind and penetrate barriers formed by immortalized human epithelial cells. It is noteworthy that this mutant retained substantial activity in the epithelial bioassay. Its ability to cross epithelial barriers seemed to be comparable with that of its homologous wild-type polypeptide. This finding is an indication that neuronal receptors and epithelial receptors are not identical. This conclusion was further reinforced by the in vivo studies. Both the wild-type and the dimutant forms of HC_{50}/A were administered by the inhalation route to mice. After intervals of time that were adequate to allow for transepithelial transport of the polypeptides, plasma samples were collected and assayed. Once again, the abilities of the wild-type and dimutant polypeptides to cross epithelial barriers were found to be comparable. The in vivo data, like the in vitro data, can be taken as evidence that neuronal receptors and epithelial receptors are not identical.

As noted in the Introduction, there are two sequences of events that underlie the mechanism of poisoning in most cases of botulism. Both events involve binding to a cell surface (epithelial cells and neuronal cells), and in both cases it is the carboxyl-terminal half of the heavy chain that mediates binding. However, there are profound differences in almost every aspect of epithelial cell handling and neuronal cell handling of the toxin (Simpson, 2004). Most obviously, epithelial cells transport unmodified toxin across the length of the cell, where the toxin is released without having caused any obvious changes in cell function. Neuronal cells deliver modified toxin (e.g., separation of heavy and light chains) to the cytosol that is proximate to the sites of binding, where the toxin dramatically alters cell function (e.g., blockade of exocytosis).

Yet more examples of differences are beginning to emerge as the details of cellular handling are becoming known. For example, there are major differences in the endocytotic events in epithelial cells and neuronal cells, as first reported

by Maksymowych and Simpson (1998) and confirmed by Couesnon et al. (2009). As now described in the present study, there are differences between the initial binding event on epithelial cells and the initial binding event on neuronal cells. The fact that the toxin depends on two such dissimilar cell types and dissimilar cellular handing mechanisms to produce its effects can only add to the extraordinary character of the molecule.

Assessment of Vaccine Activity. The HC_{50} domains of serotypes A, B, and E are being vigorously evaluated as vaccine candidates (Simpson, 2009). When administered by injection, these polypeptides evoke a robust circulating IgG response (Byrne and Smith, 2000), and when administered by the mucosal route, these polypeptides evoke both a secretory and a circulating immunoglobulin response (Ravichandran et al., 2007). These immunoglobulin responses are associated with resistance to large challenge doses of toxin.

In terms of efficacy, the HC_{50} domains show great promise as vaccines, but there is one potentially troubling aspect of HC_{50} activity. There is clear evidence that these polypeptides can bind to, and be internalized by, neuronal cells (Lalli et al., 1999). This is an undesirable property, but it is one that can be eliminated. As the data in this article demonstrate, mutated forms of BoNT HC_{50} domains can be generated that are devoid of significant ability to interact with nerve cells, but that retain significant ability to penetrate epithelial cells. Therefore, a mutated form of HC_{50} would represent a superior vaccine candidate, whether administered by the injection route or the mucosal route.

Two experiments were performed to demonstrate the efficacy of the dimutant HC_{50} domain. The first of these focused on administration of the isolated antigen, and the second focused on administration of antigen plus adjuvant. In both cases the dimutant form of HC_{50} evoked an immunoglobulin response and resistance. Furthermore, the antiserum that resulted from the dimutant immunization protocols retained the ability to bind to toxin and block its association with nerve endings. This is one of the characteristic properties of antiserum that results from immunization with the native proteins (Ravichandran et al., 2007).

In conclusion, the dimutated form of the BoNT/A HC_{50} domain possesses the desirable properties of native domains, including the ability to evoke an immune response and resistance after inhalation administration. On the other hand, this domain does not possess the undesirable property of binding and entering nerve cells. In the aggregate, these data indicate that there is a clear advantage to pursuing the dimutant HC_{50} domain as a human vaccine candidate, but no corresponding advantage to pursuing the native polypeptide as a vaccine candidate.

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Authorship Contributions

Participated in research design: Elias, Al-Saleem, Ancharski, Nasser, and Simpson.

Conducted experiments: Elias, Al-Saleem, Ancharski, Singh, Nasser, Olson, and Simpson.

Contributed new reagents or analytic tools: Elias, Singh, and Olson.

Performed data analysis: Elias, Al-Saleem, Singh, Nasser, Olson, and Simpson.

Wrote or contributed to the writing of the manuscript: Elias, Al-Saleem, Ancharski, Singh, Nasser, Olson, and Simpson.

Other: Simpson acquired funding for the research.

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